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## Relaxed Specificity of the *Bacillus subtilis* TatAdCd Translocase in Tat-Dependent Protein Secretion<sup>▽</sup>

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**Protein translocation via the twin arginine translocation (TAT) pathway is characterized by the translocation of prefolded proteins across the hydrophobic lipid bilayer of the membrane. In *Bacillus subtilis*, two different Tat translocases are involved in this process, and both display different substrate specificities: PhoD is secreted via TatAdCd, whereas YwbN is secreted via TatAyCy. It was previously assumed that both TatAy and TatCy are essential for the translocation of the YwbN precursor. Through complementation studies, we now show that TatAy can be functionally replaced by TatAd when the latter is offered to the cells in excess amounts. Moreover, under conditions of overproduction, TatAdCd, in contrast to TatAyCy, shows an increased tolerance toward the acceptance of various Tat-dependent proteins.**

The translocation of proteins across the hydrophobic membranes of cells is an essential mode of survival for all organisms. Several mechanisms have evolved to ensure this transport. Among these mechanisms, the twin arginine translocation (TAT) pathway has been shown to be capable of translocating fully folded proteins across the membrane. Proteins that depend on this translocase for their secretion are characterized by an R/K-R-X- $\phi$ - $\phi$  motif in their signal peptide, in which  $\phi$  represents a hydrophobic residue. Protein translocation via this pathway has been extensively studied mainly in the thylakoids of plants and the gram-negative bacterium *Escherichia coli* (reviewed in references 5, 20, and 25). Details on the unique mechanism of activity of the Tat pathway remain to be elucidated, although several working models have been proposed that assist in the understanding of this complex translocation machinery (9, 24, 33). In both *E. coli* and thylakoids, the Tat core complex has been shown to consist of TatA, TatB, and TatC. Of these, the TatB and TatC proteins are involved in initial substrate recognition and binding (1), whereas TatA is thought to form a protein translocation channel (10, 19). These three proteins are known to interact with and be dependent on each other, ensuring the formation of a stable core complex (21). Additionally, in *E. coli*, a fourth Tat component (TatE) has been identified that shows high similarity to TatA. This protein is able to substitute for TatA in the translocation of several Tat substrates (27).

The above-described composition of the Tat machinery is fairly consistent for the majority of thylakoidal and gram-negative Tat machineries. In gram-positive microorganisms, however, some distinct differences in the composition of the Tat

complex have been observed (17). All gram-positive Tat systems, with the exception of those in *Streptomyces* species, lack the TatB component. Moreover, most contain multiple paralogues of TatA and TatC. Recently, complementation studies with TatA of *Bacillus subtilis* showed a bifunctional activity of gram-positive TatA, as it was able to restore secretion in both a *tatA* and a *tatB* mutant of *E. coli* (4).

To obtain further insight into the working mechanism of the gram-positive Tat secretion system, including differences from and similarities to the secretion systems of gram-negative bacteria and thylakoids in chloroplasts, the gram-positive soil bacterium *Bacillus subtilis* has been adopted as a model organism for these studies. Within *B. subtilis*, three variants of the TatA protein have been identified, TatAd, TatAy, and TatAc. TatAd has been shown to form an active complex with one of the two *B. subtilis* TatC proteins, namely, TatCd. The genes encoding these proteins are located in the *phoD* operon of the *pho* regulon, which is only expressed during phosphate starvation growth conditions (16). PhoD, a phosphodiesterase of which the corresponding gene is located directly upstream of the *tatAd* gene, is the only substrate identified so far as being dependent on TatAdCd for its secretion. The second TatC component, TatCy, forms an active complex with TatAy and specifically translocates the iron-dependent peroxidase YwbN (15). The function of the third TatA protein (TatAc) is unknown, although it has been established that this protein is not required for the translocation of the two Tat-dependent substrates identified to date (15). Previous complementation studies with *E. coli* have already confirmed the difference in substrate specificity between the two Tat translocases of *B. subtilis*. In a *tat* deletion strain of *E. coli*, the secretion of the Tat-dependent trimethylamine *N*-oxide reductase TorA could only be restored when TatAdCd of *B. subtilis* was offered to the cell. Although shown to be active in the secretion of several other Tat-dependent proteins of *E. coli*, TatAyCy was unable to facilitate the secretion of TorA (4).

Even though the difference in substrate specificity between TatAdCd and TatAyCy has been clearly documented, it is so far unknown how these translocases recognize their substrates

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TABLE 1. Plasmids and strains used in this study<sup>a</sup>

Plasmids and strains	Relevant properties	Reference
<b>Plasmids</b>		
pGDL48	pGDL41 derivative lacking the <i>sipS</i> gene and containing an <i>mcs</i> ; 7.5 kb; Km <sup>r</sup>	22
pCAy	pGDL48 derivative containing the <i>tatAy</i> gene; 7.0 kb; Ap <sup>r</sup> ; Km <sup>r</sup>	15
pCCy	pGDL48 derivative containing the <i>tatCy</i> gene; 7.5 kb; Ap <sup>r</sup> ; Km <sup>r</sup>	15
pCACy	pGDL48 derivative containing the <i>tatAy-tatCy</i> operon; 7.7 kb; Ap <sup>r</sup> ; Km <sup>r</sup>	15
pCAd	pGDL48 derivative containing the <i>tatAd</i> gene; 7.0 kb; Ap <sup>r</sup> ; Km <sup>r</sup>	This study
pCCd	pGDL48 derivative containing the <i>tatCd</i> gene; 7.5 kb; Ap <sup>r</sup> ; Km <sup>r</sup>	This study
pCACd	pGDL48 derivative containing the <i>tatAd-tatCd</i> operon; 7.7 kb; Ap <sup>r</sup> ; Km <sup>r</sup>	This study
pCac	pGDL48 derivative containing the <i>tatAc</i> gene; 7.0 kb; Ap <sup>r</sup> ; Km <sup>r</sup>	This study
pDG646	Plasmid carrying the Em resistance cassette for <i>Bacillus</i> ; 4.3 kb; Ap <sup>r</sup> ; Em <sup>r</sup>	12
pDG1664	Promotes ectopic integration into the <i>thrC</i> locus; 6.0 kb; Em <sup>r</sup> ; Sp <sup>r</sup>	11
pTHR-P <sub>Ay</sub> <i>tatCy</i>	pDG1664 derivative containing a P <sub>tatAy</sub> - <i>tatCy</i> fusion; 7.5 kb; Em <sup>r</sup> ; Sp <sup>r</sup>	This study
pJAd1	pUC21 derivative containing the <i>tatAd</i> gene; Ap <sup>r</sup>	15
pJRA1	pJAd1 derivative containing flanking regions of <i>tatAd</i> disrupted by a terminator-less Em resistance cassette; 5.8 kb; Ap <sup>r</sup> ; Em <sup>r</sup>	This study
<b>Strains</b>		
<i>E. coli</i>		
MC1061	F <sup>-</sup> <i>araD139</i> $\Delta$ ( <i>ara-leu</i> )7696 $\Delta$ ( <i>lac</i> )X74 <i>galU galK hsdR2 mcrA mcrB1 rslP</i>	32
<i>B. subtilis</i>		
168	<i>trpC2</i>	18
168 X- <i>ywbN</i>	<i>trpC2 amyE::xylA-ywbN-myc</i> Cm <sup>r</sup>	15
$\Delta$ <i>tatAy</i> X- <i>ywbN</i>	<i>trpC2 tatAyCy::Sp thrC::P<sub>Ay</sub>-tatCy amyE::xylA-ywbN-myc</i> Sp <sup>r</sup> Em <sup>r</sup> Cm <sup>r</sup>	This study
$\Delta$ <i>tatCy</i> X- <i>ywbN</i>	<i>trpC2 tatCy::Sp amyE::xylA-ywbN-myc</i> Sp <sup>r</sup> Cm <sup>r</sup>	15
$\Delta$ <i>tatAyCy</i> X- <i>ywbN</i>	<i>trpC2 tatAyCy::Sp amyE::xylA-ywbN-myc</i> Sp <sup>r</sup> Cm <sup>r</sup> ; previously referred to as <i>tatAyCy::Sp</i> X- <i>ywbN</i>	15
$\Delta$ <i>tatAyCy-tatAc</i> <sub>2</sub> X- <i>ywbN</i>	<i>trpC2 tatAyCy::Sp tatAc::Tc amyE::xylA-ywbN-myc</i> Sp <sup>r</sup> Tc <sup>r</sup> Cm <sup>r</sup>	15
$\Delta$ <i>tat</i> total <sub>2</sub> X- <i>ywbN</i>	<i>trpC2 tatAyCy::Sp tatAc::Em tatAdCd::Km amyE::xylA-ywbN-myc</i> Sp <sup>r</sup> Em <sup>r</sup> Km <sup>r</sup> Cm <sup>r</sup> ; previously referred to as total- <i>tat</i> <sub>2</sub>	15
$\Delta$ <i>tatAd</i>	<i>trpC tatAd::Em</i> Em <sup>r</sup>	This study
$\Delta$ <i>tatCd</i>	<i>trpC2 tatCd::Cm</i> ; previously referred to as $\Delta$ <i>tatCd</i> (Cm)	14
$\Delta$ <i>tat</i> total	<i>trpC2 tatAyCy::Sp tatAc::Em tatAdCd::Cm</i> Sp <sup>r</sup> Em <sup>r</sup> Cm <sup>r</sup> ; previously referred to as total- <i>tat</i>	14

<sup>a</sup> Ap<sup>r</sup>, ampicillin resistance; Km<sup>r</sup>, kanamycin resistance; Em<sup>r</sup>, erythromycin resistance; Sp<sup>r</sup>, spectinomycin resistance; Cm<sup>r</sup>, chloramphenicol resistance.

and whether substrate recognition and the formation of a functional translocase are restricted to the specific TatAdCd and TatAyCy combinations only. Therefore, to shed new light on the working mechanisms and specificities of these two Tat translocases, complementation studies were performed in several *B. subtilis* *tat* mutant backgrounds, and the effect of Tat component complementation on the translocation of YwbN and PhoD in these strains was monitored. Here we show that TatAd can functionally replace TatAy when offered in excess amounts to a *tatAy* mutant *B. subtilis* strain, suggesting that TatAd and TatCy can cross-interact and that the resulting translocase is able to transport YwbN to the extracellular medium. Furthermore, our results show that the overproduction of both TatAd and TatCd facilitates the secretion of YwbN in the absence of its specific TatAyCy translocase. In contrast, no secretion of TatAdCd-dependent PhoD was observed upon the overproduction of TatAy and TatCy in the absence of the TatAd or TatCd component. These findings demonstrate that TatAdCd is more tolerant in the acceptance of Tat-dependent substrates than TatAyCy and suggest that the TatA component plays a determining role in this process.

# MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** All strains and plasmids used in this study are listed in Table 1. All strains were grown in TY (tryptone/yeast extract) medium, consisting of Bacto tryptone (1% [wt/vol]), Bacto yeast extract (0.5% [wt/vol]), and NaCl (1% [wt/vol]), unless indicated otherwise. The induction of a

phosphate depletion response was achieved by overnight growth in high-phosphate depletion medium and dilution and subsequent growth in low-phosphate depletion medium (LPDM). Both media were prepared as described by Müller et al. (23). When required, media were supplemented with erythromycin (5 µg/ml), kanamycin (10 µg/ml), chloramphenicol (5 µg/ml), tetracycline (6 µg/ml), and/or spectinomycin (100 µg/ml). To ensure the production of YwbN-myc, the expression of the corresponding gene was induced by the addition of 1% xylose to cells after entering the exponential growth phase.

**DNA cloning techniques.** All cloning techniques and the transformation of *E. coli* were performed as described by Sambrook et al. (26). The transformation of *Bacillus subtilis* was performed as described by Bron and Venema (8). All enzymes used were from Roche Molecular Biochemicals or Fermentas Life Sciences. The PCR was performed using Expand DNA polymerase (Roche) as previously described by van Dijk et al. (31).

**Construction of complementation plasmids pCAd, pCCd, pCACd, and pCac.** To construct plasmid pCAd, the *tatAd* gene was amplified from the *B. subtilis* 168 chromosome by PCR using primers JW05Ad2 (5'-ACG CGT CGA CGA ATT AAG GAG TGG-3') and JW06Ad2 (5'-GGA ATT CCG GTG TCT GCC TCA TCA GC-3'). The amplified fragment was cleaved with SalI and EcoRI and cloned into the corresponding sites of pGDL48 (22), resulting in pCAd. To construct plasmid pCCd, the *tatCd* gene was amplified by PCR using primers CdfwdSal (5'-ACG CGT CGA CGA AAG GGA GGG CTT TTT TG-3') and CdevEco (5'-GGA ATT CGA AGT CAC CGG GTG GTA CG-3'). The amplified fragment was cleaved with SalI and EcoRI and cloned into the corresponding sites of pGDL48, resulting in pCCd. To construct plasmid pCACd, the *tatAd-tatCd* region was amplified by PCR using primers JW05Ad2 (5'-ACG CGT CGA CGA ATT AAG GAG TGG-3') and CdevEco (5'-GGA ATT CGA AGT CAC CGG GTG GTA CG-3'). The amplified fragment was cleaved with SalI and EcoRI and cloned into the corresponding sites of pGDL48, resulting in pCACd. To construct plasmid pCac, the *tatAc* gene was amplified by PCR with the primers JW09Ac2 (5'-ACG CGT CGA CTT CAA TCA GGG GGA AAA GG-3') and JW10Ac2 (5'-GGA ATT CTC GGC CCA AAC GAT TTA TCC-

3'). The amplified fragment was cleaved with SalI and EcoRI and cloned into the corresponding sites of pGDL48, resulting in pC<sub>Ac</sub>.

**Construction of *Bacillus subtilis*  $\Delta$ tatAy X-ywbN.** To construct *B. subtilis*  $\Delta$ tatAy X-ywbN, the promoter region of *tatAy* was fused to the *tatCy* gene, and the resulting cassette was introduced into the *thrC* locus of the *B. subtilis*  $\Delta$ tatAyCy X-ywbN strain (15). The amplification of the upstream region of *tatAy* was realized using primers REAy-up-BamHI-F (5'-CGC GGA TCC GGA AAA CGC TTG ATC AGG ATG-3') and REAy-up-Esp3I-R (5'-CGC GTC TCG ATT TGG GCT CCT CCT TTC CC-3'). The *tatCy* gene was amplified using primers PAy-Cy-Esp3I-F (5'-CGC GTC TCG AAA TAT GAC ACG AAT GAA AGT GAA TC-3') and RECy-down-HindIII-R (5'-CCC AAG CTT CTT TGC CGT AGG GTG CAT C-3'). The amplified fragments were cleaved with Esp3I. Cleavage of the *tatCy* PCR product with Esp3I created a 5' overhang compatible with the 3' overhang of the Esp3I-cleaved PCR product carrying the *tatAy* promoter region, allowing the production of a fusion of the respective promoter region with the *tatCy* gene. The resulting product was cleaved with BamHI and HindIII and cloned into the corresponding sites of pDG1664 (11), resulting in pTHR-P<sub>AtatAy</sub>tatCy. Finally, the  $\Delta$ tatAy X-ywbN strain was obtained by the ectopic integration of the P<sub>AtatAy</sub>-*tatCy* cassette into the *thrC* locus of *B. subtilis* 168  $\Delta$ tatAyCy X-ywbN via a double crossover event. The correct integration of the P<sub>AtatAy</sub>-*tatCy* cassette was verified by analyzing its growth on minimal medium (8) with or without the addition of threonine.

**Construction of *Bacillus subtilis*  $\Delta$ tatAd.** To construct the  $\Delta$ tatAd strain, a terminator-less erythromycin resistance cassette was amplified from pDG646 with primers Pery-Hind-F (5'-CCC AAG CTT CTC TAG AGG ATC CTT TAA CTC TGG C-3') and Ery-Hind-R (5'-CGG AAG CTT TTA CTT ATT AAA TAA TTT ATA GCT ATT GAA AAG AG-3'). The resulting PCR fragment was cleaved with HindIII and ligated into the corresponding site of pJAd1 (15). The resulting plasmid, pJRad, was checked for the correct orientation of the Em<sup>r</sup> cassette using a SacI restriction analysis and used for the transformation of *B. subtilis* 168. Finally, the *B. subtilis* 168  $\Delta$ tatAd strain was obtained by a double crossover recombination event between the Em<sup>r</sup>-disrupted *tatAd* gene on pJRad and the chromosomal *tatAd* gene. Consequently, the downstream *tatCd* gene was placed under the control of P<sub>ery</sub>.

**Protein techniques.** To detect YwbN-myc and PhoD, the medium and cellular fractions were prepared as described previously (16). After separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred to a polyvinylidene difluoride membrane (Molecular Probes, Inc.) as described previously (30). YwbN was visualized with specific antibodies against the C-terminal myc epitope (Clontech Laboratories, Inc.) and horseradish peroxidase-conjugated goat anti-mouse antibodies (Amersham Biosciences) according to the manufacturer's instructions. PhoD was visualized with specific antibodies against PhoD (kindly provided by J. Müller) and horseradish peroxidase-conjugated goat anti-rabbit antibodies (Amersham Biosciences) according to the manufacturer's instructions.

## RESULTS

**TatAd can functionally replace TatAy.** To study the level of functional compatibility between Tat components of *Bacillus subtilis*, the secretion of the TatAyCy-dependent substrate YwbN was monitored in several *tat* deletion strains, complemented by different *B. subtilis* Tat components expressed from a constitutive promoter (production increased approximately 5- to 10-fold [data not shown]). For this purpose, the X-ywbN-myc cassette and plasmids encoding different Tat components and bearing operons were introduced into *tat* mutant strains as described before (15). Next, overnight TY cultures of wild-type and *tat* deletion strains, with or without complementation plasmids, were diluted 100-fold in fresh TY medium. The strains were grown to exponential growth phase, at which time YwbN-myc production was induced by the addition of 1% xylose. After 3 h of induction, the cells were harvested by centrifugation, separating the cellular fractions from the extracellular medium fractions. In order to analyze the production and secretion of YwbN-myc, SDS-PAGE samples were prepared as described in Materials and Methods and analyzed by SDS-PAGE, Western blotting, and immunodetection with antibod-

ies raised against the c-Myc epitope. Importantly, an analysis of the presence of this protein in the cellular fractions demonstrated the production of YwbN-myc in all the strains studied (data not shown). Notably, no significant accumulation of the YwbN precursor (pre-YwbN) was observed in these fractions.

TatAy has been shown to be a key component in the secretion of YwbN, as deletion of this vital component completely abolishes YwbN secretion (Fig. 1A, lane  $\Delta$ Ay). Since the deletion of *tatCy* results in the same effect (Fig. 1B, lane  $\Delta$ Cy) and because the genes are organized in an operon, it has been suggested that these two components together form an active complex, in which both play a critical role in the secretion of YwbN (15). Notably, the absence of (pre-)YwbN in these fractions implies that these *tat* mutant strains do not show an increased sensitivity to lysis, as was also described previously for *B. subtilis* (14) and in contradiction to what was observed for *tat* mutants of *E. coli* (29). Moreover, an analysis of the presence of the cytoplasmic protein DnaJ demonstrated the absence of this protein in medium fractions of wild-type and *tatAy* mutant strains, indicating that *tat* mutant strains are not more sensitive to lysis (data not shown). To study the putative complementation of *tatAy* or *tatCy* deletions, individual Tat components were introduced into the *tatAy* and *tatCy* mutant strains expressed from pGDL48 derivatives. YwbN secretion into the extracellular medium was visualized using c-Myc-specific antibodies.

As expected, YwbN secretion was restored in both the *tatAy* and the *tatCy* deletion strains by the production of plasmid-borne TatAy or TatCy, respectively (Fig. 1A, lane Ay, and Fig. 1B, lane Cy), as well as by complementing the *tatAy* and *tatCy* deletion strains with the complete TatAyCy complex (Fig. 1A and B, lane AyCy). Strikingly, plasmid-borne TatAd is also able to restore YwbN secretion in the *tatAy* deletion strain (Fig. 1A, lane Ad). It should be noted that during these experimental growth conditions in rich TY medium, the endogenous *tatAd* and *tatCd* genes are not expressed. Therefore, these observations suggest that TatAd is able to form an active complex with endogenous TatCy, resulting in the secretion of YwbN at levels comparable to those during complementation with plasmid-borne TatAy. In addition, and as expected on the basis of the results with complementing TatAd, the production of TatAd and TatCd (Fig. 1A, lane AdCd) also efficiently restores YwbN secretion. In contrast to TatAd forming a functional Tat complex with TatCy, TatAy seems to be unable to form functional complexes with TatCd, as TatCd is unable to complement the deletion of *tatCy* (Fig. 1B, lane Cd). However, the introduction of TatAdCd into the *tatCy* mutant strain does restore YwbN secretion (Fig. 1B, lane AdCd), suggesting that the TatAdCd complex is able to translocate YwbN. Notably, in this situation the TatAy component is still available for YwbN secretion as well. However, the assumption that TatAdCd alone is capable of YwbN transport is confirmed by the results shown in Fig. 1C, lanes AdCd, demonstrating that even in the absence of both TatAy and TatCy (the  $\Delta$ tatAyCy strain), the constitutive production of TatAdCd can restore the secretion of YwbN.

**TatAdCd can only aid in the secretion of YwbN when over-produced.** The above-described phenomenon of TatAdCd being functional in the secretion of YwbN was only observed



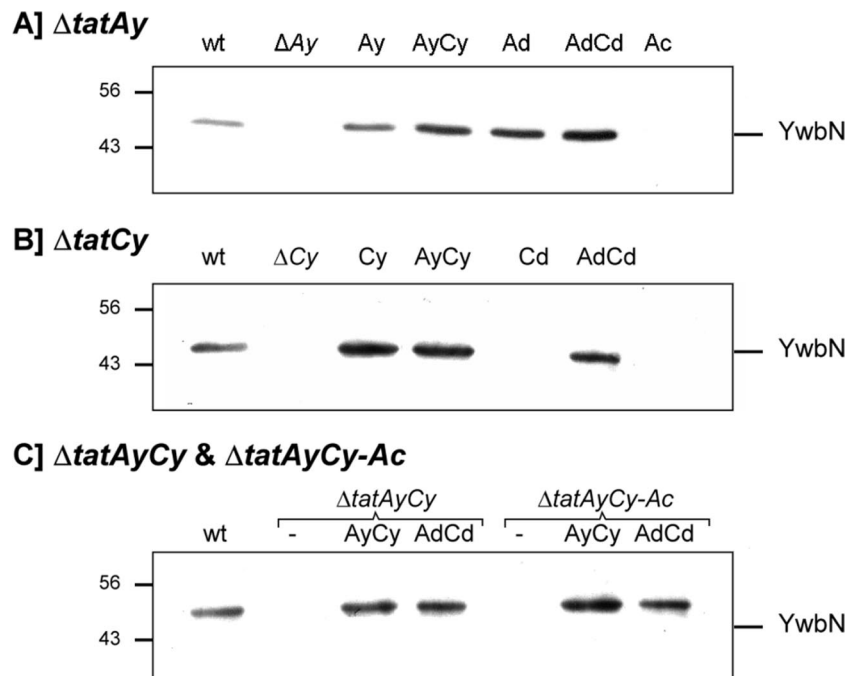


FIG. 1. YwbN secretion in several *tat* mutant backgrounds, complemented by various Tat components. The secretion of YwbN-myc (indicated on the right) into the extracellular medium was visualized using specific antibodies against the C-terminal myc tag. The size of the protein is indicated by marker bands on the left (in kDa). The secretion of YwbN-myc in the *B. subtilis* 168 X-ywbN-myc parental strain is shown in the first lane (wt). (A) *Bacillus subtilis*  $\Delta$ *tatAy* (lane  $\Delta$ Ay) with xylose-induced YwbN-myc complemented by plasmid-borne TatAy (lane Ay), TatAyCy (lane AyCy), TatAd (lane Ad), TatAdCd (lane AdCd), and TatAc (lane Ac). (B) *Bacillus subtilis*  $\Delta$ *tatCy* (lane  $\Delta$ Cy) with xylose-induced YwbN-myc complemented by plasmid-borne TatCy (lane Cy), TatAyCy (lane AyCy), TatCd (lane Cd), and TatAdCd (lane AdCd). (C) *Bacillus subtilis*  $\Delta$ *tatAyCy* ( $\Delta$ AyCy) and  $\Delta$ *tatAyCy-tatAc* ( $\Delta$ AyCyAc) with xylose-induced YwbN-myc (X-ywbN) complemented by plasmid-borne TatAyCy (lane AyCy) or TatAdCd (lane AdCd). Noncomplemented strains are indicated with a – symbol.

when TatAd and TatCd components were expressed from pGDL48 derivatives. Since the genes are constitutively expressed from the Em<sup>r</sup> promoter of pDGL48, the production of the TatAd and TatCd components is most likely increased. This is also demonstrated by the fact that introducing constitutively expressed TatAyCy into the  $\Delta$ *tatAy* and  $\Delta$ *tatCy* mutant strains mostly results in an increase in YwbN secretion (Fig. 1A, B, and C, lanes AyCy) compared to that in the parental strain (Fig. 1A, B, and C, lanes wt). To examine whether TatAdCd is also able to complement *tatAy* and/or *tatCy* deletions at wild-type expression levels of endogenous *tatAd* and *tatCd*, we grew the  $\Delta$ *tatAy* and  $\Delta$ *tatCy* mutant strains in a low-phosphate medium, thereby initiating the expression of *tatAdCd* from the chromosome (3). The cells were grown in the presence of 1% xylose for 3 h after reaching exponential phase to induce the expression of *ywbN-myc*, and the secretion of YwbN was monitored. As is clearly shown in Fig. 2, YwbN cannot be detected in the extracellular medium of cells in which the *tatAy* and/or *tatCy* genes are deleted (Fig. 2, lanes  $\Delta$ Ay,  $\Delta$ Cy, and  $\Delta$ AyCy). This implies that under growth conditions with wild-type expression levels of *tatAd* and *tatCd*, the TatAdCd translocase is specifically dedicated to the export of PhoD and, putatively, other as-yet-unknown TatAdCd-specific substrates. This is consistent with earlier findings by Jongbloed et al. (15).

**TatAyCy components are unable to complement the absence of TatAd and TatCd.** The results described above imply that certain Tat components can cross-interact to form an active

translocase (i.e., TatAd with TatCy) and that a certain degree of substrate tolerance, at least by *B. subtilis* TatAdCd translocases, is accepted. This possibility was further investigated by performing complementation studies with the  $\Delta$ *tatAd* and  $\Delta$ *tatCd* mutant backgrounds. *B. subtilis* 168  $\Delta$ *tatAd*,  $\Delta$ *tatCd*, and  $\Delta$ *tat* total, in which all Tat components are absent, strains were grown in LPDM as described in Materials and Methods to induce the expression of the *phoD* operon, including *phoD*, *tatAd*, and *tatCd*. Shortly after reaching transition phase, the cells were separated from the growth medium by centrifugation as described in Materials and Methods. Next, the presence of PhoD in the cellular fractions of the above-mentioned

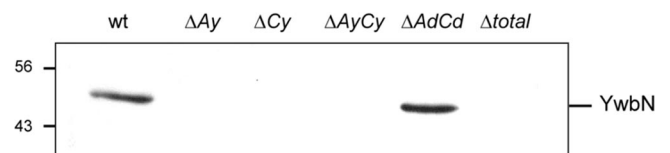


FIG. 2. Secretion of YwbN in several *tat* mutant backgrounds when grown in LPDM. Cells were grown in LPDM as described in Materials and Methods. The expression of *ywbN-myc* was induced by the addition of 1% xylose as described in Materials and Methods. YwbN-myc in the extracellular medium (indicated on the right) was visualized using specific antibodies against the C-terminal myc tag. The size of the protein is indicated by marker bands on the left (in kDa).  $\Delta$ Ay, *B. subtilis* 168  $\Delta$ *tatAy* X-ywbN;  $\Delta$ Cy, *B. subtilis* 168  $\Delta$ *tatCy* X-ywbN;  $\Delta$ AyCy, *B. subtilis* 168  $\Delta$ *tatAyCy* X-ywbN;  $\Delta$ AdCd, *B. subtilis* 168  $\Delta$ *tatAdCd* X-ywbN;  $\Delta$ total, *B. subtilis* 168  $\Delta$ *tat* total<sub>2</sub> X-ywbN.

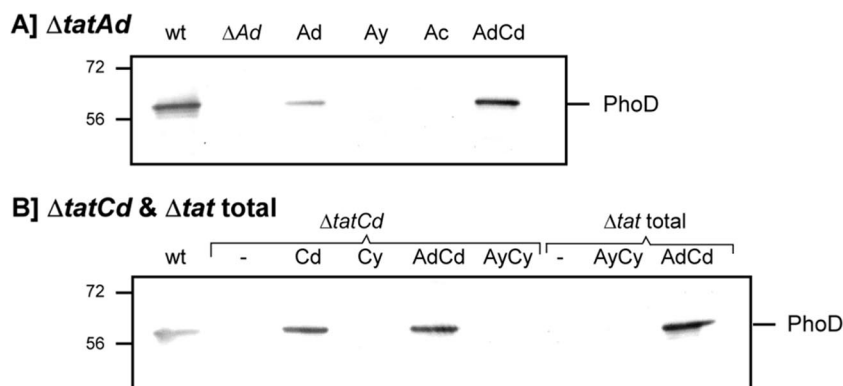


FIG. 3. PhoD secretion in several *tat* mutant backgrounds, complemented by various Tat components. Cells were grown in LPDM to induce the expression of *phoD* and *tatAdCd*. The secretion of PhoD (indicated on the right) into the extracellular medium was visualized using specific antibodies against PhoD. The size of the protein is indicated by marker bands on the left (in kDa). Secretion of PhoD in the parental strain *Bacillus subtilis* 168 is indicated in the first lane (wt). (A) *B. subtilis* 168  $\Delta tatAd$  (lane  $\Delta Ad$ ) complemented by plasmid-borne TatAd (lane Ad), TatAy (lane Ay), TatAc (lane Ac), or TatAdCd (lane AdCd). (B) *B. subtilis* 168  $\Delta tatCd$  and *B. subtilis* 168  $\Delta tat$  total complemented by plasmid-borne TatCd (lane Cd), TatCy (lane Cy), TatAdCd (lane AdCd), or TatAyCy (lane AyCy). Noncomplemented strains are indicated with a – symbol.

strains was verified by an analysis of these samples by SDS-PAGE and Western blotting and subsequent immunodetection using specific antibodies against PhoD (data not shown). The secretion of PhoD into the extracellular medium was monitored by applying a similar approach. As shown in Fig. 3, PhoD is only secreted into the medium when the absence of the TatAd and TatCd components is restored by the reintroduction of the respective TatAd and/or TatCd components expressed from a plasmid (Fig. 3A and B, lanes Ad, Cd, and AdCd). In contrast, the introduction of plasmid-borne TatAy, TatCy, or TatAyCy does not result in PhoD secretion (Fig. 3A and B, lanes Ay, Cy, and AyCy). Although the complementation by plasmid-borne TatAd in a  $\Delta tatAd$  mutant background is less efficient compared to wild-type secretion levels (Fig. 3A, lane Ad), these experiments were repeated several times and showed a reproducible secretion pattern. In none of the cases was complementation by TatAy observed. These results show that, unlike previously described for TatAdCd, the TatAyCy complex is not able to complement the absence of TatAdCd components and suggest that TatAyCy is less substrate tolerant than TatAdCd.

**TatAc does not actively participate in Tat-dependent protein translocation.** The third *tatA* gene of *B. subtilis*, *tatAc*, is monocistronic and has no TatC counterpart. So far no functional activity for TatAc has been documented. Since the *tatAc* gene is not associated with any *tatC* gene on the chromosome, it seems unlikely that this third TatA component of *Bacillus subtilis* is involved in the secretion of Tat-dependent proteins. This hypothesis is confirmed by the inability of TatAc to complement the absence of TatAy in the secretion of YwbN or of TatAd in the secretion of PhoD (Fig. 1A, lane Ac, and Fig. 3A, lane Ac).

## DISCUSSION

The two Tat translocases of *Bacillus subtilis* are known to display different substrate specificities, in which YwbN is specifically secreted via TatAyCy and PhoD via TatAdCd (15). To obtain more insight into the specificity determinants of Tat-

dependent protein secretion in *B. subtilis*, the ability of plasmid-borne Tat components (solely or in combination, i.e., TatAdCd or TatAyCy) to complement different *tat* deletions was studied. Which specific molecular interactions determine substrate specificity remains to be elucidated.

By monitoring the secretion of epitope-tagged YwbN during complementation in the *tatAy* and *tatCy* deletion strains by different Tat components, we were able to show that the absence of TatAy can be complemented by plasmid-borne TatAd (Fig. 1A, lane Ad). In contrast, the absence of *tatCy* could not be complemented by *tatCd* expression from the pCCd plasmid (Fig. 1B, lane Cd). Nevertheless, it seems that YwbN translocation can be facilitated by a TatAdCd complex in the absence of the TatAy and/or TatCy components, since YwbN was shown to be secreted in  $\Delta tatCy$  and  $\Delta tatAyCy(Ac)$  mutant strains that are complemented by the overproduction of the TatAd and TatCd components (Fig. 1B, lane AdCd, and Fig. 1C, lanes AdCd). In previous studies, it was shown that TatAdCd is able to translocate the *E. coli* Tat substrate TorA in the *E. coli* *tat* null mutant strain  $\Delta ABCDE$  (4). Notably, complementation by TatAdCd in *E. coli* as well as in *B. subtilis* was only observed when excess amounts of the TatAd and TatCd components were offered to the cells (Fig. 1 and 2) (4). It is therefore feasible that the TatAdCd complex may have a low affinity for the YwbN substrate when produced at wild-type levels, which cannot be detected with the techniques used in this study. The overexpression of TatAd and TatAdCd can overcome this low affinity, which results in a relaxed substrate specificity. Taken together, these results provide new insights into the substrate specificity and complex formation of Tat components of *B. subtilis*. The fact that TatAd can complement the absence of TatAy for the secretion of YwbN suggests that it is likely that TatAd forms an active complex with TatCy, provided that TatAd is present in excess amounts. In contrast, a TatAdCy complex seems to be unable to facilitate the secretion of PhoD, as the overproduction of TatCy in a  $\Delta tatCd$  mutant background, in which TatAd is still produced, does not result in the presence of PhoD in the extracellular medium of this strain (Fig. 3B, lane Cy). Likewise, TatAy is unable to form

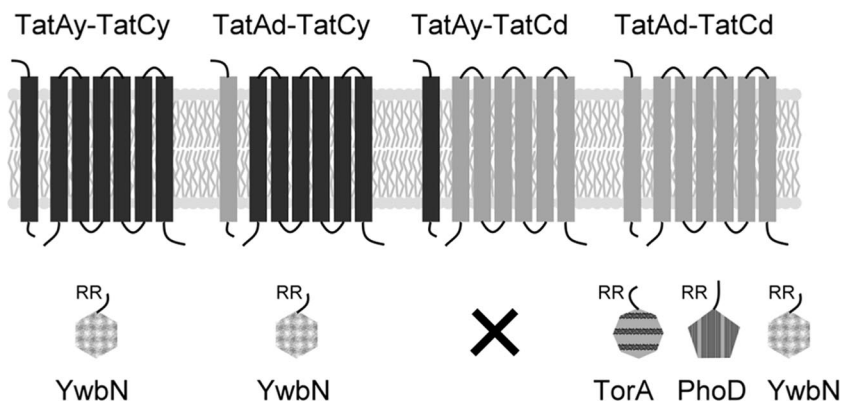


FIG. 4. Tat component interactions and activities. The Tat proteins studied are represented by rectangles in a membrane, connected by intracellular and extracellular loops. TatA has a single membrane spanning domain with a predicted outside N terminus and inside C terminus. TatC has six transmembrane spanning domains and a predicted in-in topology. TatAy and TatCy are represented by black rectangles, whereas TatAd and TatCd are represented by light-gray rectangles. The naturally present TatAyCy complex is specific in the secretion of YwbN (hexagon) harboring an RR consensus motif in the signal peptide. In the absence of TatAy, complementation with plasmid-borne TatAd results in the secretion of YwbN into the extracellular environment. In contrast, results indicative of a TatAyCd complex that is active in the secretion of either YwbN or PhoD could not be obtained (indicated with a black x). Finally, overproduction of the TatAdCd complex in a rich medium can facilitate the secretion of YwbN in *B. subtilis* and TorA (octagon) in *E. coli* (4). Additionally, at wild-type levels, produced as well as overproduced TatAdCd complexes secrete PhoD (pentagon) in *B. subtilis*.

an active complex with TatCd for the secretion of YwbN or PhoD (Fig. 1B, lane Cd) or PhoD (Fig. 3A, lane Ay), even when TatCd is overproduced. This ambiguous behavior of Tat components can only be explained by yet-to-be-identified specificity determinants in the amino acid sequence of the separate Tat components and/or Tat substrate signal peptides that promote successful Tat protein interactions, substrate recognition, and pre-protein transport in some cases (export of YwbN by TatAdCy) but not in other cases (export of PhoD by TatAdCy or export of YwbN by TatAyCd).

In previous studies, it was shown that the third *B. subtilis* TatA component, TatAc, is not required for the translocation of YwbN or PhoD (15). Accordingly, we now show that TatAc, despite its production in excess amounts, is unable to complement for the absence of TatAy or TatAd in the secretion of YwbN or PhoD, respectively (Fig. 1A, lane Ac, and Fig. 3A, lane Ac). It is conceivable that TatAc does not play any part in Tat-dependent protein translocation. The fact that TatAc does not have a TatC counterpart supports that theory. On the other hand, we cannot exclude the possibility that TatAc, like TatE of *E. coli*, provides an additional pool of TatA molecules that might be needed under conditions of high Tat production. Moreover, also the existence of TatAc-specific substrates can still not be excluded. In that case, the transport of these putative substrates has to be accomplished in conjunction with TatCd and/or TatCy.

With respect to the working mechanism of the Tat machinery of *E. coli* and thylakoids, it has been proposed that a TatBC complex is involved in the initial recognition and binding of Tat-dependent substrates (1, 7, 9) and that subsequently TatA proteins are recruited to form the translocation channel (6). Recent results of studies concerning gram-positive Tat complex formation in *E. coli* suggest a similar model for the activity of *B. subtilis* Tat complexes (4). Considering the fact that *B. subtilis* does not contain a TatB analogue, it would be plausible to assume that TatCy is involved in the recognition and binding

of pre-YwbN and that subsequently a separate TatAy complex is recruited that will be responsible for the translocation of the YwbN protein. This proposed working model for YwbN secretion by TatAyCy differs from the recently proposed working model for the TatAdCd-dependent translocation of PhoD by Westermann et al. (33) and Schreiber et al. (28). These authors favor a model in which cytosolic TatAd is involved in the initial recognition and binding of the double arginine-containing signal peptide of the PhoD precursor (pre-PhoD), after which this TatAd-pre-PhoD complex is targeted to a membrane-associated TatCd complex. This complex then assists in the membrane insertion of the cytosolic TatAd-pre-PhoD complex and subsequent pre-PhoD translocation. Taken together, these two models either contradict or supplement each other. Our complementation data suggest that the two Tat translocases of *B. subtilis* operate in a different manner, at least at the level of Tat substrate specificity. This hypothesis is illustrated in Fig. 4. Since we were able to show that TatAd can be involved in the secretion of both PhoD and YwbN, it is conceivable that this component is responsible for the tolerant character of the TatAdCd translocon during overexpression conditions in rich medium. In order to explain the observations described for our complementation studies, we propose, in concordance with the earlier findings of Westermann et al. (33), that TatAd can be actively involved in the recognition and binding of random Tat signal sequences, after which it can interact with membrane-bound TatCd. In contrast to TatAd, TatCd is very specific in complex formation with TatAd only, since TatAy was shown to be unable to form an active complex with TatCd, at least for the secretion of YwbN. However, TatAd is not restricted to its own counterpart, TatCd, but is also capable of forming an active complex with TatCy. Since this was only observed for the secretion of YwbN, and not for PhoD, we propose that TatCy is the specificity determining factor in the TatAyCy complex and thus responsible for recognizing the signal sequence of YwbN (in a TatAd-pre-YwbN



complex or separately). After recognizing and binding pre-YwbN, TatCy will subsequently form an active translocation complex with either TatAd or TatAy. This model is only applicable when there are excess amounts of TatAd present, since lower (wild-type) production levels (i.e., under the conditions of phosphate depletion) of TatAd do not lead to a YwbN-exporting TatAd-TatCy machine. Under these conditions, a clear substrate specificity of both Tat complexes is observed: TatAyCy being specifically involved in YwbN secretion and TatAdCd being specifically active in PhoD secretion.

Topology predictions of TatCy show that this protein most likely forms four intracellular loops and three extracellular loops that are connected by six transmembrane domains. Which part of the TatCy protein is involved in the recognition and binding of YwbN is so far unknown. Studies with *E. coli* TatC provided some clues concerning the role of conserved residues in the first two cytoplasmic loops in substrate recognition, since conservative substitutions of these residues resulted in a complete block in protein translocation (2, 13). It is possible that the same regions, or even residues, within the TatCy protein are involved in the recognition of and/or binding to pre-YwbN. We are currently pursuing this line of research further.

Finally, the specific cross-interactive nature of TatAdCd when produced in excess amounts leads to the hypothesis that the TatAdCd translocase is less specific in its affinity for Tat substrates than TatAyCy. This could consequently result in a preference of the TatAdCd translocase over the TatAyCy translocase as a candidate for the commercial production of prefolded heterologous proteins.

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